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RESEARCH ARTICLE

Polyphenols and antimicrobial activity in extracts of *Lippia alba* (Mill.)

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Abstract: Total polyphenols content was evaluated in ethanolic and aqueous extracts of leaves and inflorescences of *Lippia alba* (Mill.), from plants cultivated in the Chaco province, northeastern of Argentina. The extracts were obtained by percolation, infusion and decoction. Total phenols content was determined by Folin Ciocalteu reagent, flavonoids content was determined in Al-complex form and the free radical scavenging activity was determined by DPPH radical. The antimicrobial screening was carried out by bioautography and in the active extracts the minimal inhibitory concentration and minimal bactericidal concentration was determined by the disc diffusion method. Results in terms of phenols content, flavonoids content and DPPH radical scavenging were higher in ethanolic extracts than in the aqueous ones. There were differences statistically significant in phenols and flavonoids contents of the extracts obtained with extraction solvents of different polarity. The aqueous extracts obtained by infusion presented smaller phenols content, higher flavonoids content and better free radical scavenging activity in comparison with the extracts obtained by decoction. Only ethanolic extracts presented antimicrobial activity and they were more active against Gram-positive bacteria.

Keywords: antibacterial activity; antioxidant activity; plants extracts; total phenols.

Introduction

Verbenaceae family is widely distributed in regions of tropical and subtropical climate, from Central to South America, as well as in Africa. It consists of approximately 250 species of herbs, shrubs and small trees (Braga et al. 2005). *Lippia alba* (Miller) N.E. Brown (Verbenaceae) is an aromatic herb widely recognized in folk medicine, and its essential oil is used in the industry and cosmetic (Bandoni 2003).

In folk medicine, it is attributed sedative, digestive, carminative, antihypertensive and antispasmodic properties to this specie. Also, this plant is used for stomach colic, fever, as anti-inflammatory and for rheumatic pains. An infusion of the roots is used against nausea, chill, cough and bronchitis, to cure wounded skin and as syrup against the cough and bronchitis (Cicció and Ocampo 2006; Di Stasi 2002; Hennebelle et al. 2008). The leaves are employed in infusion or decoction, for treatment of gastric illness, diarrhea, fever, asthma, cough and as a tranquilizing (Sena Filho et al. 2006).

The chemical composition of the essential oil of *L. alba* depends sensibly on the geographical origin of the plant, the conditions of its cultivation, the age and the part of the plant used, and on some other geobotanics and genetic factors (Olivero-Verbel et al. 2009; Ricciardi et al. 1999; Stashenko et al. 2004). The phytochemical variability in this specie has given origin to a classification in different types considering the majority components (Ricciardi et al. 2000).

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The chemical composition of methanolic extracts of *L. alba* pre and post extraction of essential oil, evidences flavonoids, steroids, tannins, saponins, coumarins and lactones metabolites (Yara Varón et al. 2007). Also, there are some studies about secondary metabolites, total phenols and antioxidant activity in methanolic and ethanolic extracts of *L. alba* (Naznin and Hasan 2009; Nuñez et al. 2008; Nuñez et al. 2010).

Moreover, the essential oil of this specie demonstrates more activity against Gram-positive bacteria (Nogueira et al. 2007; Vera et al. 2007); as well as against *Candida albicans* (ATCC 14053) and *Escherichia coli* (ATCC 25992) (Vera et al. 2007). Ethanolic and methanolic extracts of roots of this specie present antimicrobial activity against *Staphylococcus aureus* (ATCC 6538P and ATCC 6538) and *Klebsiella pneumoniae* (ATCC 10031) (Sena Filho et al. 2006). Chloroformic, acetonic, ethanolic extracts of roots have antimicrobial activity against stumps of *S. aureus*, *Micrococus luteus*, *Bacillus subtilis*, *Mycobacterium smegmatis*, *C. albicans* and *Monilia sitophila* (Hennebelle et al. 2008; Aguiar et al. 2008). Ethanolic, cloroformic and n-hexane extracts and essential oil serves as antibacterial (it inhibits growth of *Vibrio parahaemolyticus*) and antimicotic (*Aspergillus niger*) (Ara et al. 2009; Henao et al. 2011).

Therefore, the purpose of this report is the comparison of total polyphenols content and antioxidant and antimicrobial activities of aqueous and ethanolic extracts of *L. alba*.

Materials and methods

Plant material

The plants were obtained by micropropagation (Sansberro et al., 2006) and were cultivated under cover in the province of Chaco, northeast region of Argentina. The fresh leaves and inflorescences were collected during the summer of the 2010 and 2011 years. The vegetal material was washed, dried 5 to 7 days at room temperature; it was processed in a knives mill Dalvo® until a smaller particle size to 350 µm.

Samples preparation

The powered samples of the plant were extracted with hot water and to boiling water (as infusion and decoction at 5 %, respectively) and with ethanol of 70° (for simple percolation, relationship power plant and extractive solvent, 1:1). Then these extracts were filtered and stored at -20 °C.

Total phenols determination

The assay was determined by Singleton et al. method (1999) with modifications. It was used fifty microliters of aqueous extracts without dilution and ethanolic extract diluted in ethanol 70° (1:10). To each tube it was added 1,950 µL of distilled water, 200 µL of Folin-Ciocalteu reagent (Sigma-Aldrich®, 1:5 with water) and 800 µL of sodium carbonate (16%). The determination was carried out after 30 minutes in darkness using a spectrophotometer Beckman DU® 640B at 760 nm and this was contrasted with the blank.

The calibration curve was determined in connection with gallic acid as a reference substance (0.5 mg/mL) and using volumes between 20 and 80 µL.

Total flavonoids determination

The assay was determined by Lock et al. method (2006) with modifications. Each extract was used in the same conditions that previous assay. To the sample, 30 µL, it was added 60 µL of aluminium nitrate 10 % w/v and 60 µL of potassium acetate 1 M. This dilution was mixed with 2,850 µL of distilled water. It was kept at room temperature for 20 minutes; the absorbance of the mixture reaction was measured at 415 nm.

The calibration curve was determined in connection with quercetine solution as reference reagent (2.7 mg/mL) and using volumes between 15 and 55 µL.

Antioxidant activity

The assay was determined by Brand-William method (1995) with modifications using DPPH radical (Sigma-Aldrich®). It was used a blank of ethanol 70° and a control with 750 µL of DPPH (0.0728 mM) and 2,250 µL of ethanol 70°. The reaction used 50 µL of extract samples in same conditions that previous assays; it was added with 750 µL of DPPH and ethanol 70° to complete 3 ml of final volume. After 10 minutes the absorbance of DPPH reaction was measured at 517 nm.

The free radicals scavenging activity is the necessary concentration of extracts to reach 50 % of bleaching of DPPH radical (IC_{50}). The DPPH concentrations were calculated from the experimental calibration curve. The inhibition percentage of DPPH was calculated from:

% DPPH = [(Absorbance control – Absorbance sample)/ Absorbance control] x 100

In which Absorbance control was with DPPH initial and Absorbance sample was the reaction between extracts and DPPH after 10 minutes.

Antimicrobial activity

Microorganisms

The following reference strains were included in the study: *Staphylococcus aureus* (ATCC 29213), *S. aureus* (ATCC 25923), *S. epidermidis* (ATCC 12228), *Enterococcus faecalis* (ATCC 29212), *E. coli* (ATCC 35218) and *Pseudomonas aeruginosa* (ATCC 27853). Clinical isolates of *S. aureus* (F13) and (F29), *Proteus mirabilis* (F304) and *Morganella morganii* (F339) were obtained from Hospital of Clinical Dr. Nicolás Avellaneda, San Miguel of Tucumán city (province of Tucumán, Argentina).

Disc diffusion method

The qualitative determination was assessed by Bauer et al. method (1966). Cell suspension ($1.5x10^8$ CFU/mL) was standardized by adjusting optical density to 0.08-0.1 at 625 nm. Petri dishes were filled with Agar Müeller–Hinton (MHA) medium and inoculated with microorganism test. Filter paper discs (6 mm diameter) were added with 30 µg/mL phenolic compounds for crude extracts. Ampicillin (10 µg) and gentamicin (120 µg) (Laboratories Britania, Argentina) were used as positive controls. Discs with 20 µl of ethanol 70° or sterile distilled water were used as negative controls. Bacterial growth inhibition was determined as the diameter of the inhibition zones around the discs. The determinations were made in duplicate.

Bioautography

It was developed by the technique of Nieva-Moreno et al. (1999) using thin-layer chromatography. A spot containing 30 µg phenolic compounds of each extract was seeded onto TLC plate (Kieselgel 60 F254 0.2 mm, Merck) The plates covered with 3mL of brain–heart infusion medium (BHI with 0.6 % agar) containing 10^5 CFU of *S. aureus* (ATCC 25923) and *P. aeruginosa*, it were incubated at 35 °C for 16-20 hours. The plates were sprayed with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium- Sigma-Aldrich®) solution (2.5 mg/mL) in PBS (10 mM sodium phosphate buffer, pH 7, with 0.15 M NaCl). The cellular viability was evaluated by means of the reduction of MTT salt that showed a yellow colour in the inhibition of microbial growth areas (Zampini et al., 2007).

Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations

These assays were according to CLSI method, 2006. MIC values were determined by two assays: serial agar macrodilution and broth microdilution method. In MBC determination was used the microdilution method. The extracts were dried and resuspended in dimethyl sulfoxide (DMSO).

Agar macrodilution method

Dilutions of crude extracts (range concentration between 62.5 and 1000 µg of phenolic compounds/mL) were prepared. Three hundred microliters (300 µL) of each diluted extract was added to 9.7 mL of MHA medium. After cooling and drying, the plates were inoculated in spots with 2 µl of each bacterial cell suspension (5×10^4 CFU) and incubated aerobically for 16-

20 hours at 35°C. A growth control of each tested strain was included. Controls of DMSO were also carried out. MIC was defined as the lowest concentration of extract at which a colony was not observed after incubation.

Broth microdilution method

This test was performed in sterile 96-well microplates. The extracts were transferred in order to obtain serial dilutions of the original extract. The inoculum (100 µl) containing 5×10^5 CFU was added to each well. A number of wells were reserved in each plate for sterility control (without added inoculate), inoculate viability (without added extract), and solvent control (DMSO). Plates were incubated at 35 °C for 16-20 hours. Bacterial growth was indicated by the presence of turbidity and a pellet on the well bottom. MIC was defined as the lowest concentration of extract that had restricted growth to a level no macroscopically visible.

To establish MBC, 10 µl of each culture medium was removed from each well with no visible growth and inoculated in MHA plates. After 16-20hours of incubation at 35 °C, the number of surviving organisms was determined. MBC was defined as the lowest extract concentration at which 99.9% of the bacteria had been killed. These experiments were carried out in duplicate.

Statistical analysis

The results were expressed as mean value ± standard deviation. The significant differences among the values obtained for each test were analyzed with Statgraphics for Windows version 5.1 by ANOVA using multiple contrasts test of ranges and Kruskall-Wallis test. The differences among groups were considered significant with $p< 0.05$.

Results

Total Phenols Content

This test was determined with a calibration curve of gallic acid as reference substance where the value of r^2 was of 0.9951.

Table 1: Phenols and Flavonoids content in *Lippia alba* extracts.

Determination	Infusion	Decoction	Ethanolic Extract
Phenols Content	2.226 ±0.078	2.296 ± 0.089	40.929 ± 0.557
Flavonoids Content	1.614 ±0.121	1.301 ± 0.074	22.761 ± 0.708

Data of phenols (in equivalent milimols of gallic acid/liter of extract) and date of flavonoids (in equivalent milimols of quercetine/liter of extract).

Statistically, the total phenols content was different among ethanolic extract and aqueous extracts ($p<0.047$), but there was not significant difference among the aqueous extracts.

Total Flavonoids content

The determination of total flavonoids content used quercetine as reference substance with a calibration curve that showed a value of r^2 was of 0.9904.

The total flavonoids content was smaller in the aqueous extracts compared with ethanolic extract, such as observed in the Table 1. The values of total flavonoids presented significant differences among the three extractive samples ($p<0.027$).

Antioxidant activity

The inhibition percentage of DPPH was calculated and the comparison among the extracts is presented in Table 2.

Table 2: Comparison of IC50 volume and antioxidant activity of *L. alba* extracts**.**

Samples	IC50 Volumen	Concentration EAG
Infusion	26.01 ± 1.65	0.339 ± 0.045
Decoction	35.79 ± 0.99	0.499 ± 0.013
Ethanolic extract	15.85 ± 3.15	0.227 ± 0.024
Reference: gallic acid	28.87 ± 0.26	0.085 ± 0.0007

Data of volume (in microliters of sample) and concentration of *L. alba* extracts for bleaching of radical DPPH (in equivalent miliMolar of gallic acid/miliMolar DPPH).

There was significant difference of Fisher (LSD) among the three samples as much in the values of volume as in total phenols content expressed as equivalent of gallic acid. Furthermore, it was found smaller free radicals scavenging capacity in the aqueous extracts that in etanolics extracts.

Antimicrobial activity

Direct bioautography and disc diffusion method demonstrated that the ethanolic extracts were active against Gram-positive but aqueous extracts were not active at the concentrations assayed. The halos values were between 8 and 10 mm for Gram-positive bacteria. As evaluating criterion equal halos or superior 10 mm were considered significant for antimicrobial activity, such as the observed in the different species of *S. aureus*.

Minimal inhibitory concentration and minimal bactericidal concentration

Ten microorganisms were tested and the results are summarized in Table 3.

Table 3: MIC and MBC of *L. alba* ethanol extract against pathogens bacteria.

Bacteria	MIC	MBC
Gram-positive		
Staphylococcus aureus (ATCC 29213)	125	250
Staphylococcus aureus (ATCC 25923)	125	250
Staphylococcus aureus (13)	125	500
Staphylococcus aureus (29)	125	250
Staphylococcus epidermidis (ATCC 12228)	125	250
Enterococcus faecalis (ATCC 29212)	125	1000
Gram-negative		
Pseudomonas aeuruginosa (ATCC 27853)	550	>1000
Proteus mirabilis (F304)	550	>1000
Morganella morganii (F339)	600	>1000
Escherichia coli (ATCC 35218)	800	>1000

MIC was the lowest concentration of phenols/mL of extract at which colony was not observed after incubation. MBC was the lowest concentration of phenols/mL of extract at which 99.9% of the bacteria have been killed.

The ethanolic extract presented antimicrobial activity against all microorganism tested. The effects observed on *Staphylococcus* species were bactericidal (MBCs were within a two-fold dilution of the MICs) and it was bacteriostatic for *E. faecalis* and Gram-negative strains (which were the most resistant).

Discussion

The smallest phenols and flavonoids contents in aqueous extracts could be related with the chemical nature of the phenolic compounds (Arranz Martínez S., 2010), the polarity of extraction solvent (Eloff et al, 2005), the solubility of each component in the solvent and their sensibility to Folin-Ciocalteau reagent (Singleton; Rosi, 1965).

The free antiradical scavenging activity could be attributed to some characteristics of their phenolics compound. A bigger activity is related with small phenols molecules, including flavonoids and phenolic acids. Moreover, the phenols of high molecular weight (tannins) had bigger ability to capture free radicals and their effectiveness depends on the molecular weight, aromatic rings number and nature of the substitution of hydroxyls group and the functional specific groups (Manian et al., 2008).

Also, some phenolic antioxidants reacting strongly to the Folin-Ciocalteu reagent may not react to the DPPH free radicals (Yang et al., 2007). This could justify the smallest free radical scavenging capacity of the aqueous extracts even when the volume used for the purification is similar to the gallic acid solution (reference substance).

Smallest activities of the extracts against Gram-negative bacteria were observed in coincidence with other reports (Holetz et al., 2002; Aguiar et al., 2008). This can be justified because the Gram-negative bacteria present an external surrounding membrane that restricts the diffusion of hidrofobics compounds through the lipopolisaccharide of cellular membrane. Besides, the periplasmatic space contains enzymes that can prevent strange molecules from introducing from outside (Duffy and Power, 2001; Laciar et al., 2009).

Our results with better antimicrobial activity against Gram-positive bacteria have agreement with those mentioned by other reports in *L. alba* (Aguiar et al., 2008, Ara et al., 2009). The differences in the results could be attributed to the extraction of different components because of the employment of different extraction methods and solvents of different polarity.

In conclusion, ethanolic extracts present a bigger phenols content and flavonoids content than the aqueous extracts. The ethanolic extract also showed bigger antioxidant activity and this could be related to the characteristics of phenolic compounds extracted with ethanol. The antibacterial activity of ethanolic extract against some microorganisms was in accordance to the folk use of this plant for infectious diseases and then the ethanolic extract could be used in infections caused mainly by Gram- positive bacteria.

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